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Microbial inoculant effects on silage and *in vitro* ruminal fermentation, and microbial biomass estimation for alfalfa, bmr corn, and corn silages

Francisco E. Contreras-Govea[a,*], Richard E. Muck[b], David R. Mertens[b], Paul J. Weimer[b]

[a] *Plant and Environmental Sciences Department, New Mexico State University-Agricultural Science Center at Artesia, 67 E. Four Dinkus Road, Artesia, NM 88210, United States*

[b] *U.S.D.A., Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, United States*

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ABSTRACT

Whole crop third cut alfalfa, brown mid-rib (bmr) corn, and corn were chopped and inoculated with one of four microbial inoculants used. Uninoculated silage was the control treatment. Each crop was ensiled in four mini-silos (1 L glass jars) per treatment. All silos were fermented for 60 days at room temperature (22 °C), and then they were opened and analyzed for fermentation products, fiber constituents and N fractions. A fraction of wet silage was ground with a blender for 30 s. *In vitro* gas production was measured in 160 ml sealed serum vials at 3, 6, 9, 24, and 48 h using the wet ground silage. At 9 and 48 h, rumen fluid was analyzed for volatile fatty acids (VFA) and microbial biomass yield (MBY). In all the three crops, the four inoculants produced only minor changes in pH and fermentation products during ensiling. Of the variables measured, soluble nonprotein N fractions were the characteristics most often affected by some inoculants. At 9 h incubation, *in vitro* gas production and VFA did not differ between control and inoculated silages, but MBY did. Among crops, alfalfa and corn silages had higher MBY than did bmr corn silage. Among inoculants, three of the four inoculated silages produced more MBY than did control. At 48 h, alfalfa silage produced higher MBY than did corn or bmr silage, and two of the inoculated silages had more MBY than did the control. There was no inoculant by crop interaction. Results suggest that some silage inoculants are capable of altering rumen fermentation, even in cases where effects on silage fermentation are small, and that this effect may be linked to better preservation of crop protein during ensiling.

1. Introduction

Microbial silage inoculants containing lactic acid bacteria have long been used to improve silage fermentation. Inoculants were originally used to reduce pH and to avoid, or decrease, the risk of a clostridial fermentation by the native bacterial population (Wilkinson et al., 2003). Muck and Kung (1997) conducted a literature review on the effects of microbial inoculants on silage fermentation. They reported that, compared with uninoculated silage, 60% of the studies reported that inoculated silages reduced pH, promoted lactic acid production, and reduced ammonia formation. In an evaluation of 14 microbial

Abbreviations: ADF, acid detergent fiber expressed inclusive of residual ash; bmr, brown mid-rib; CP, crude protein; DM, dry matter; FAA, free amino acids; IVTDMD, *in vitro* true DM digestibility; LAB, lactic acid bacteria; MBY, microbial biomass yield; aNDF, amylase-treated neutral detergent fiber expressed inclusive of residual ash; NDFD, aNDF digestibility; NPN, soluble nonprotein N; VFA, volatile fatty acids; WSC, water-soluble carbohydrates.

* Corresponding author. Tel.: +1 575 748 1228; fax: +1 575 748 1229.
E-mail address: fecontre@nmsu.edu (F.E. Contreras-Govea).

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inoculants on alfalfa silage, Filya et al. (2007) reported that most, but not all, of the inoculants reduced pH and increased lactic acid production relative to uninoculated silage.

In addition, research indicates that microbial inoculation affects not only plant fermentation but also animal performance as indicated by increased milk yield, weight gain and/or feed intake (Kung and Muck, 1997; Kung et al., 2003). Kung et al. (1993) studied the effects of microbial inoculants on silage fermentation and milk yield by lactating dairy cows. Two experiments were conducted with corn silage, with and without inoculation with lactic acid bacteria, in which silage fermentation characteristics were similar between inoculated and uninoculated silage. However, in both studies inoculated silage increased milk production and dry matter (DM) intake compared with uninoculated silage. In a literature review, Weinberg and Muck (1996) observed that many commercial inoculants improve silage fermentation, but effects on animal performance did not always coincide with changes in silage fermentation. They hypothesized that some inoculants may have a probiotic effect in the rumen. Kung and Muck (1997) also reported that silage inoculation improved DM intake in 28%, weight gain in 53%, and milk yield in 47% of the studies where these indices of animal performance were measured. Across only the studies where inoculation increased milk yield, the inoculated silages increased milk production 1.36 kg/cow/day compared to untreated silages. They speculated that this could be due to increased propionate concentrations in the rumen, lower silage ammonia and free amino acid (FAA) concentrations, higher concentrations of silage peptides, higher digestibility and/or inhibition of growth of undesirable microorganisms. They further speculated that animal performance improvements were due to the microbial inoculants, particularly lactic acid bacteria, surviving fermentation and entering the rumen alive. Weinberg et al. (2003, 2004) demonstrated that lactic acid bacteria can survive *in vitro* rumen incubation, shifting *in vitro* fermentation and volatile fatty acid (VFA) composition. In addition, Gollop et al. (2005) found that many silage inoculants have antibacterial activity and that this activity is imparted to inoculated silages.

In vitro gas production techniques have been developed to estimate rate and extent of ruminal DM degradation (Schofield and Pell, 1995; Rymer et al., 1998). Additionally, these techniques have demonstrated that VFA produced by ruminal microorganisms during *in vitro* fermentation is positively related to gas production (Blümmel et al., 1997b). Blümmel et al. (1997b) proposed a simple technique to estimate microbial biomass yield (MBY) from *in vitro* ruminal fermentation as:

$$\text{MBY} = \text{truly digested sample} - \text{apparently digested sample}$$

where true digestibility and apparent digestibility were determined as described by Blümmel et al. (1997b), without using sodium sulfite but with heat stable amylase in the neutral detergent as described by Van Soest et al. (1991). A recent study conducted by Muck et al. (2007) with 14 microbial inoculants in alfalfa silage concluded that silage inoculation had an effect on *in vitro* ruminal gas and VFA production. Some inoculated silages produced more gas and VFA than the untreated silages, and some displayed reduced gas and VFA production compared to control silages. However, information about the relationships among *in vitro* gas production, VFA yield and MBY, and information about how microbial silage inoculants could impact any of these three products, is limited in the literature. Therefore our objective was to determine the effects of four inoculants on silage fermentation characteristics and *in vitro* ruminal production of gas, microbial biomass, and VFA.

2. Material and methods

2.1. Ensiling procedure

Third cutting alfalfa harvested at 1/10 bloom (August 20, 2004), brown midrib (bmr) corn (Mycogen F-697, Mycogen Seeds, Indianapolis, IN, USA) harvested at 1/2 milk line (September 28, 2004), and corn (Lemke 7028, Lemke Seed Farms Inc., Mequon, WI, USA) harvested at 2/3 milk line (September 23, 2004) were chopped at a theoretical length of cut of 10 mm with a conventional forage harvester. Each crop was ensiled with five treatments: 1) No inoculant (Control), 2) *Lactobacillus plantarum* and *Enterococcus faecium* (LP–EF; Pioneer 1174, Pioneer Hi-Bred International Inc., Des Moines, IA, USA), 3) *L. plantarum* (LP; Ecosyl MTD/1, Ecosyl, Stokesley, North Yorkshire, UK), 4) *Lactobacillus pentosus* (LPe; Agri-King, Agri-King, Fulton, IL, USA) and 5) *Lactococcus lactis* (LL; Genus PLC, Basingstoke, UK). Three of the four inoculants were chosen because of *in vitro* gas production results of a previous study (Muck et al., 2007) in which LP–EF inoculated silage had one of the highest rates of *in vitro* gas production, LP inoculated silage was similar or less than the untreated control, and LPe inoculated silage was consistently less than the control. The LL was chosen because it produces only L (+) isomer of lactic acid whereas the other inoculants produce a mix of the two isomers. While previous studies have shown no differences in total and individual VFA from ruminal fermentation of the two lactate isomers (Weiss et al., 2003), effects of isomer on other end products of ruminal fermentation have not been measured. All crops were ensiled separately the day of cutting in 1.0 L glass jars (Weck, Wher-Oftlingen, Germany) at a density of 500 g/L, using four jars per treatment. Each inoculant was applied at a 10^6 cfu/g fresh weight. Each inoculant was diluted to achieve an application rate of 1 g solution per 100 g crop, and each silo contained 500 g of crop. The control was sprayed with 5.0 g of distilled water. Over the course of packing each crop, four samples were collected for initial characteristics. Silos were stored for 60 days at room temperature (~22 °C), and fermentation was stopped by freezing the silos to −20 °C until the silages were analyzed.

A 20 g sample was taken from each silo, diluted 10 fold on a mass basis with autoclaved, distilled water, and macerated for 30 s in a high speed blender. The diluted sample was analyzed by a pour plate technique for lactic acid bacteria using Lactobacilli MRS agar (Difco™ 288210, Becton, Dickinson and Co., Sparks, MD, USA). The remaining diluted sample was

filtered through 4 layers of cheesecloth, and pH was measured immediately with a pH meter (Thermo Orion Model 525, Thermo Fisher Scientific, Waltham, MA, USA). Two 20 mL aliquot samples were placed in two separate 50 mL polypropylene centrifuge tubes. One sample was used for ammonia and free amino acids determination by adding 5 mL of 250 g/L trichloroacetic acid, centrifuging for 20 min at 25,100 × *g* at 4 °C, and pouring the supernatant into a 20 mL scintillation vial. The second 20 mL sample was centrifuged for 20 min at 25,100 × *g* at 4 °C, and the supernatant was transferred to a scintillation vial and frozen for later analysis of fermentation products and water soluble carbohydrates (WSC; Dubois et al., 1956). Fermentation products (*i.e.*, succinate, lactate, acetate, propionate, butyrate, and ethanol) were determined using high performance liquid chromatography (HPLC; Muck and Dickerson, 1988). The HPLC system used a refractive index detector (RID-6A, Shimadzu Corp., Kyoto, Japan) and a Bio-Rad Aminex HPX-87H column (Bio-Rad Lab., Hercules, CA, USA) heated to 42 °C.

Two additional silage samples were collected for moisture analysis by freeze-drying. These sub-samples were ground through a 1 mm screen Wiley mill and used for the determination of N by a Leco FP-2000A N analyzer (Leco Corp., St. Joseph, MI, USA), sequential extraction of acid detergent fiber (ADF) after extraction of aNDF (with heat stable amylase and sulfite) using an ANKOM fiber analyzer (Ankom Technology Corp., Fairport, NY, USA) and *in vitro* true DM digestibility (IVTDMD) using the Daisy II System (ANKOM Technology Corp., Fairport, NY, USA). Both aNDF and ADF are expressed inclusive of residual ash. Ammonia, free amino acids, and soluble nonprotein N (NPN) concentrations (Broderick et al., 2004) were determined using flow-injection (Lachat Quik-Chem 8000 FIA; Lachat Instruments, Milwaukee, WI, USA). Peptide concentration was estimated by subtracting free amino acids and ammonia concentrations from NPN concentration (Licitra et al., 1996).

The remaining silage from each mini-silo was chopped using a commercial Robot Coupe food processor (Robot Coupe, Inc., Joliet, IL, USA) for 30 s, achieving a particle size of 1–4 mm. Chopped fresh silage was vacuum sealed in a 30 cm × 40 cm plastic bag and frozen to −20 °C for later analysis of *in vitro* rumen fermentation and gas production.

2.2. Gas production procedure

In vitro gas production followed the procedure of Muck et al. (2007). Briefly, 1.0 g of wet ground silage was collected randomly, weighed and placed in volume calibrated 160 mL serum bottles, which were assigned randomly for each *in vitro* run (Wheaton, Millville, NJ, USA). Each bottle received 17.1 mL buffer solution, and was placed in a water bath (39 °C) and purged with CO_2 for 30 min. Each bottle then received 0.9 mL of reducing solution (*i.e.*, 6.25 g each of cysteine HCl and $Na_2S{\bullet}9H_2O$/L), and was capped and placed in a warm room (39 °C) for 45 min. *In vitro* ruminal inoculum was prepared by following the procedure described by Weimer et al. (2005). Rumen fluid was collected from four fistulated lactating dairy cows fed a total mixed ration diet that contained 0.30 alfalfa silage, 0.30 corn silage, 0.30 corn grain, and 0.10 soybean meal, plus supplemental vitamins and minerals. After the samples were reduced, 12 mL of inoculum were added to each serum bottle, which was then capped with a butyl rubber stopper, sealed with an aluminum crimp, and kept at 39 °C. Gas pressure was measured at 3, 6, 9, 24, and 48 h with a digital pressure gauge (model SEDPGB0015PG5, SenSym, Milpitas, CA, USA). Gas production values were corrected by subtracting gas production from blank bottles (*i.e.*, buffer solution, reducing solution, and ruminal inoculum), and then adjusted proportionally by the ratio of gas production from a dried alfalfa standard in the run to its average gas production from prior analyses. Each *in vitro* run analyzed all of the silages of a single crop, and there were two *in vitro* runs for each crop.

At 9 and 48 h in each *in vitro* run, one sample bottle per silo was opened. The contents were analyzed for pH and VFA using the same HPLC system described above. Microbial biomass yield (MBY) was calculated as previously described using the procedure of Blümmel et al. (1997b) with the modification to express MBY in mg/100 mg of truly digested sample as:

$$\text{MBY} = \frac{\text{truly digested sample} - \text{apparently digested sample}}{\text{truly digested sample}}$$

The MBY measurements at 9 h and 48 h were chosen because 9 h was approximately the end of linear gas production and 48 h represented 0.95–0.98 of the relative gas production at 96 h in an earlier *in vitro* study with undried silages (Muck et al., 2007). Thereafter, microbial decay would increasingly influence microbial biomass yields. In addition, Hall and Weimer (2007) reported maximum microbial crude protein (CP) at 8 h of *in vitro* fermentation. The 48 h measurement corresponds with typical solids retention times in the rumen (Weimer et al., 2009).

2.3. Statistical analysis

The silage fermentation data were analyzed as a split-plot randomized complete block design using the PROC mixed procedure of SAS Inc. (2001), with crop as whole plot, inoculant treatment (subplot) and crop by inoculant interaction as fixed effects and silo and silo by crop interaction as random effects. Differences among means were tested using the adjusted Tukey test with significance declared at $P<0.05$.

In vitro VFA and microbial biomass estimates at 9 and 48 h data were analyzed using average values of both *in vitro* runs for each replicate silo per crop and analyzed as a split plot randomized complete block design using the PROC mixed procedure of SAS Inc. (2001), with crop as whole plot, inoculant treatment (subplot) and crop by inoculant interaction as fixed effects and silo and silo by crop interaction as random effects. Differences among means were tested using the adjusted Tukey test with significance declared at $P<0.05$.

Table 1
Alfalfa, bmr corn (bmr), and corn (CS) characteristics before ensiling.

	Alfalfa	SE	Corn silage		SEM
			bmr	CS	
DM (g/kg)	395	1.5	352[b]	392[a]	10.5
pH	6.12	0.024	6.30[a]	5.89[b]	0.072
Lactic acid bacteria, log(cfu/g)	7.81	0.028	6.18[b]	7.35[a]	0.235
aNDF (g/kg DM)	327	8.4	362	317	29.8
ADF (g/kg DM)	248	9.7	184	148	16.9
WSC (g/kg DM)	52.0	0.78	65.9[b]	87.3[a]	5.00
IVTDMD (g/kg DM)	836	2.8	883	883	10.4
CP (g/kg DM)	225	4.6	62.5[a]	56.2[b]	1.33
FAA-N (g/kg total N)	83.6	3.30	45.7	41.2	1.63
NH_3-N (g/kg total N)	8.2	0.50	16.5	13.5	0.97
NPN (g/kg total N)	223	13.3	157[a]	97[b]	13.7
Peptides (g/kg total N)	131	13.9	94[a]	43[b]	11.7

WSC = water soluble carbohydrates; IVTDMD = *in vitro* true DM digestibility; FAA-N = free amino acid N; NH_3-N = ammonia N; NPN = non protein N.
[a,b] Corn silage means within a row with different superscripts differ ($P<0.05$).

The equation used to fit gas production data (adjusted for blanks) was:

$$y = A(1 - e^{-ct})$$

as described by Blümmel et al. (2003), where y is the gas volume at time t, A is the asymptotic value of gas production and c is the first order fractional rate constant of gas production. One-way analysis of variance was conducted to test statistical difference on A and c parameters among treatments within each crop (SAS Inc., 2001).

3. Results

3.1. Silage fermentation characteristics

Characteristics of all crops before ensiling indicated high quality with low fiber concentrations, high IVTDMDs, DM ranging from 352 to 395 g/kg, and low NPN and ammonia N (Table 1). The natural populations of lactic acid bacteria were high on all crops, particularly the alfalfa, providing substantial competition for the inoculants which were applied at 6.0 log(cfu/g crop). The bmr corn was higher than the corn in pH, CP, NPN and peptides.

After fermentation, DM, aNDF, ADF, CP, IVTDMD, aNDF digestibility (aNDFD), and WSC concentrations were similar between control and inoculated silages (Table 2), and there were no crop by inoculant interactions with the exception of DM where the interaction was in the bmr corn silage where LL had higher DM (348 g/kg) than control and other inoculated treatments (328 g/kg). There were no differences in DM between treatments in the other crops.

The NPN, peptide and ammonia fractions expressed on a total N basis were affected by microbial inoculant (Table 3), and there were crop by inoculant interactions for those three constituents indicating differences in response to the inoculants across the three crops. In contrast, FAA was not affected by inoculant or a crop by treatment interaction. In both NPN and peptides, only LP was lower than the control among crops, but in bmr corn silage only LL had lower NPN and peptide concentrations than control. In ammonia, LP was higher than control and LL averaged over the three crops. However, the ranking of inoculant treatments by ammonia value from highest to lowest was not consistent by crop.

Table 2
Nutritive characteristics of alfalfa (AS), bmr corn (bmr), and corn (CS) silages inoculated with one of four microbial inoculants.[a]

	DM (g/kg)	aNDF (g/kg DM)	ADF (g/kg DM)	CP (g/kg DM)	WSC (g/kg DM)	IVTDMD (g/kg DM)	aNDFD (g/kg aNDF)
Crop							
AS	379[A]	384[B]	307[A]	219[A]	5.3[B]	797[B]	473[B]
CS	363[B]	368[B]	194[C]	57[C]	14.6[A]	851[A]	596[A]
bmr	332[C]	411[A]	235[B]	63[B]	13.8[A]	845[A]	621[A]
SEM	2.5	8.3	6.4	1.2	0.67	5.5	13.0
Inoculant							
Control	360	385	242	113	11.4	835	568
LP–EF	362	373	239	114	10.9	839	570
LP	353	399	250	112	9.9	827	566
LPe	356	390	247	114	11.3	831	566
LL	360	392	248	111	12.6	823	547
SEM	2.8	10.9	6.7	1.5	0.74	7.1	15.3

WSC = water soluble carbohydrates; IVTDMD = *in vitro* true DM digestibility; aNDFD = aNDF digestibility.
Control = no inoculant; LP–EF = *Lactobacillus plantarum–E. faecium*; LP = *L. plantarum*; LPe = *L. pentosus*; LL = *Lactococcus lactis*.
[a] There were no crop by inoculant interactions for any characteristic except dry matter as detailed in the text.
[A–C] Means within a column with different superscripts differ ($P<0.05$).

Table 3
Soluble nitrogen fractions (g/kg total N) of alfalfa (AS), bmr corn (bmr), and corn (CS) silages inoculated with one of four microbial inoculants.

	Treatment					SEM
	Control	LP–EF	LP	LPe	LL	
AS						
NPN	459^{ab}	458^{ab}	420^{b}	518^{a}	448^{b}	25.3
Peptides	85^{d}	103^{d}	106^{d}	148^{c}	96^{d}	21.1
FAA-N	337	318	276	330	312	15.2
NH_3-N	37.9^{e}	37.4^{e}	37.2^{e}	40.0^{e}	40.3^{e}	1.45
bmr						
NPN	504^{a}	513^{a}	472^{a}	475^{a}	427^{b}	25.3
Peptides	215^{b}	211^{b}	182^{bc}	201^{bc}	158^{bc}	21.1
FAA-N	238	250	235	224	221	15.2
NH_3-N	52.4^{ab}	51.3^{bc}	55.5^{a}	50.3^{bc}	48.0^{cd}	1.45
CS						
NPN	488^{a}	458^{ab}	401^{b}	521^{a}	530^{a}	25.3
Peptides	199^{bc}	166^{bc}	104^{d}	205^{bc}	271^{a}	21.1
FAA-N	244	243	247	265	214	15.2
NH_3-N	45.0^{d}	48.5^{cd}	50.0^{bc}	52.2^{b}	45.1^{d}	1.45

Control = no inoculant; LP–EF = *Lactobacillus plantarum–E. faecium*; LP = *L. plantarum*; LPe = *L. pentosus*; LL = *Lactococcus lactis*; NPN = soluble nonprotein N; FAA-N = free amino acid N; NH_3-N = ammonia N.
[a–e]Means within a row with different superscripts differ ($P<0.05$).

3.2. Silage fermentation profile

Effects of microbial inoculants on pH and fermentation were relatively minor among the crops (Table 4). Inoculant treatment affected only acetic and succinic acid concentrations, but there were crop by inoculant interactions for lactic, acetic and succinic acids. pH and ethanol were affected by crop, but not by inoculant or the interaction. With lactic acid, differences in concentration between the highest and the lowest treatments were small and ranged from 4.8 g lactic/kg DM for corn silage to 12.5 g/kg DM for alfalfa silage. The control was similar to the inoculants in the two corn silages whereas in alfalfa the LP was lower than the control, the primary cause of the interaction. Other inoculants in the alfalfa were similar to the control.

As with lactic acid, differences in acetic acid between the highest and the lowest concentrations within a crop were small, 5.9–7.9 g acetic acid/kg DM. The LL and the control had the lowest concentrations across crops, whereas LPe, LP and LP–EF were higher and similar. The crop by inoculant interaction occurred primarily because in the alfalfa, LP had the lowest acetic acid concentration while in the bmr corn silage LPe and LL had the lowest acetic acid concentrations.

Succinic acid was detected in all crops and was the fourth most abundant fermentation product. Across the three crops, LP and LL had lower concentrations than the control. The LL was similar to the control in the alfalfa and corn silages, and LP was similar to the control in the bmr corn silage.

Table 4
Fermentation profile (g/kg DM) of alfalfa (AS), bmr corn (bmr), and corn (CS) silage inoculated with one of four microbial inoculants.

	Treatment					SEM
	Control	LP–EF	LP	LPe	LL	
AS						
pH	4.58	4.59	4.61	4.61	4.60	0.013
Lactic acid	76.5^{a}	74.3^{a}	67.0^{b}	72.7^{ab}	79.5^{a}	2.30
Acetic acid	37.1^{ab}	38.3^{a}	34.0^{b}	41.9^{a}	39.2^{a}	1.48
Ethanol	6.9	4.4	5.3	7.5	7.3	1.80
Succinic acid	6.4^{b}	6.8^{ab}	5.9^{c}	6.9^{a}	6.4^{b}	0.14
bmr						
pH	3.88	3.87	3.92	3.89	3.88	0.013
Lactic acid	73.7^{a}	76.4^{a}	71.5^{ab}	74.6^{a}	69.5^{b}	2.30
Acetic acid	22.0^{cd}	21.3^{cd}	24.4^{c}	19.6^{de}	18.5^{de}	1.48
Ethanol	12.1	12.3	11.0	15.0	12.4	1.80
Succinic acid	2.8^{d}	2.8^{d}	2.9^{d}	3.0^{d}	1.6^{e}	0.14
CS						
pH	3.82	3.84	3.84	3.85	3.85	0.013
Lactic acid	45.0^{c}	43.3^{c}	46.5^{c}	44.1^{c}	48.1^{c}	2.30
Acetic acid	18.0^{de}	21.8^{cd}	23.4^{c}	23.0^{c}	15.9^{e}	1.48
Ethanol	18.5	22.7	15.1	21.5	20.1	1.80
Succinic acid	1.2^{ef}	1.1^{f}	0.8^{f}	0.8^{f}	1.2^{ef}	0.14

Control = no inoculant; LP–EF = *Lactobacillus plantarum–E. faecium*; LP = *L. plantarum*; LPe = *L. pentosus*; LL = *Lactococcus lactis*.
[a–f]Means within a row with different superscripts differ ($P<0.05$).

Table 5
In vitro ruminal asymptotic gas production (A, mL/g DM) and first order fractional rate constant of gas production (*c*, / h) of alfalfa (AS), bmr corn (bmr), and corn (CS) silage inoculated with one of four microbial inoculants.

	A	*c*
AS		
Control	174	0.110
L. plantarum–E. faecium	172	0.112
L. plantarum	176	0.105
L. pentosus	176	0.108
L. lactis	174	0.109
SEM	2.1	0.0021
P	0.715	0.327
bmr		
Control	281	0.097
L. plantarum–E. faecium	267	0.100
L. plantarum	263	0.098
L. pentosus	275	0.100
L. lactis	270	0.100
SEM	6.2	0.0026
P	0.374	0.940
CS		
Control	250[b]	0.151
L. plantarum–E. faecium	274[ab]	0.126
L. plantarum	288[a]	0.118
L. pentosus	289[a]	0.121
L. lactis	301[a]	0.122
SEM	10.4	0.0146
P	0.042	0.571

[a,b]Means within a column with different superscripts differ ($P<0.05$).

3.3. *In vitro* gas production

There were no differences in asymptotic gas production (*A*) and first order fractional rate constants of gas production (*c*) values among inoculants except in corn silage (Table 5) where all inoculated silages except LP–EF had higher asymptotic gas production than control. The first order fractional rate constant of gas production did not differ among treatments in corn silage.

3.4. Microbial biomass yield and volatile fatty acids

3.4.1. 9 h *in vitro* fermentation

There were no differences in VFA concentrations, acetate:propionate (A:P) ratio, and gas production among inoculant treatments, but there were differences among crops ($P<0.05$, Table 6). However, microbial biomass yield was affected by both crop ($P<0.0001$) and inoculants ($P<0.0016$, Table 6), but there was no crop by inoculant interaction ($P<0.633$). Total VFA (54.5 mM) and A:P ratio (3.19) were the highest in alfalfa silage, but total VFA in corn silage (52.6 mM) was not lower than that of alfalfa silage. The MBY was similar between alfalfa (39.3 mg/100 mg TD) and corn silages (39.5 mg/100 mg TD), but higher than for bmr corn silage (32.7 mg/100 mg TD). Alfalfa silage had the lowest gas production (114 mL/g DM) of the three crops. Among inoculants, LPe, LL, and LP produced, on average, 8.0% more MBY than control and LP–EF.

Table 6
In vitro produced volatile fatty acids, ruminal microbial biomass yield, and gas production at 9 h incubation in alfalfa (AS), bmr corn (bmr), and corn (CS) silage inoculated with one of four microbial inoculants.

	Acetate (mM)	Propionate (mM)	Butyrate (mM)	VFA (mM)	A:P	MBY (mg/100 mg TD)	Gas (mL/g DM)
Crop							
AS	37.7[a]	11.9[b]	4.9[c]	54.5[a]	3.19[a]	39.3[a]	114[c]
CS	31.5[b]	13.9[a]	7.9[a]	52.6[a]	2.25[b]	39.5[a]	196[a]
bmr	27.8[c]	12.5[b]	6.8[b]	47.1[b]	2.21[b]	32.7[b]	168[b]
SEM	0.59	0.31	0.16	1.07	0.035	0.63	2.7
Inoculant							
Control	32.2	12.9	6.6	51.7	2.53	35.4[b]	158
LP–EF	31.6	12.4	6.3	50.3	2.51	35.5[b]	158
LP	31.8	12.3	6.3	50.5	2.61	37.9[a]	157
Lpe	32.9	12.9	6.6	52.4	2.58	39.0[a]	162
LL	33.1	13.3	6.9	53.3	2.53	38.0[a]	162
SEM	0.74	0.33	0.17	1.30	0.036	0.72	3.1

A:P = acetate/propionate ratio; MBY = microbial biomass yield; TD = truly digested dry matter; Control = no inoculant; LP–EF = *Lactobacillus plantarum–E. faecium*; LP = *L. plantarum*; LPe = *L. pentosus*; LL = *Lactococcus lactis*.
[a–c]Means within a column with different superscripts differ ($P<0.05$).

Table 7
In vitro produced volatile fatty acids, rumen microbial biomass yield, and gas production at 48 h incubation of alfalfa (AS), bmr corn (bmr), and corn (CS) silage inoculated with one of four microbial inoculants.

	Acetate (mM)	Propionate (mM)	Butyrate (mM)	VFA (mM)	A:P	MBY (mg/100 mg TD)	Gas (mL/g DM)
Crop							
AS	50.1[a]	15.9[b]	7.1[c]	73.0[b]	3.26[a]	34.8[a]	172[c]
CS	49.8[a]	18.0[a]	11.0[a]	78.9[a]	2.81[b]	20.2[b]	285[a]
bmr	45.5[b]	18.2[a]	9.9[b]	73.5[b]	2.50[c]	18.5[c]	272[b]
SEM	0.45	0.27	0.12	0.74	0.043	0.43	3.2
Inoculant							
Control	48.2	16.8	9.4[a]	74.4	2.90	23.5[c]	236
LP–EF	48.3	17.7	9.4[a]	75.4	2.86	23.7[c]	239
LP	49.0	17.1	8.9[b]	75.0	2.91	25.2[ab]	243
Lpe	47.8	17.7	9.4[a]	74.9	2.81	24.4[bc]	248
LL	48.9	17.6	9.6[a]	76.1	2.79	25.8[a]	249
SEM	0.59	0.35	0.16	0.96	0.051	0.56	4.2

A:P = acetate/propionate ratio; MBY = microbial biomass yield; TD = truly digested dry matter; Control = no inoculant; LP–EF = *Lactobacillus plantarum–E. faecium*; LP = *L. plantarum*; LPe = *L. pentosus*; LL = *Lactococcus lactis*.
[a–c]Means within a column with different superscripts differ ($P<0.05$).

3.4.2. 48 h in vitro fermentation

At 48 h of *in vitro* fermentation (Table 7), similar effects among crops occurred as at 9 h. However, at 48 h, corn silage had the highest total VFA (78.9 mM), and MBY was 80% higher in alfalfa silage than in the corn silages. In contrast, alfalfa silage produced 38% less gas than the corn silages (Table 7).

Among inoculants, LP was the only inoculated silage with lower butyrate than control and other inoculated silages (Table 7). At 48 h, only LL and LP produced more MBY than did the control. On average, LL and LP produced 8.1% more MBY than did LP–EF and control, with no differences in gas production.

For three products of *in vitro* fermentation at 48 h (*i.e.*, acetate, total VFA, and gas), there were crop by inoculant interactions. In acetate this occurred because LP had the lowest value in bmr corn silage (43.2 *versus* 45.7–46.6 mM/L for the other treatments; SEM 1.01 mM/L) and the highest value in corn silage (53.0 *versus* 46.6–50.5 mM/L). With total VFA, the interaction was also caused by differences between the corn silages. In bmr corn silage, LP was lower than in the other treatments (69.8 *versus* 73.9–75.4 mM/L; SEM 1.66 mM/L) whereas in corn silage the control was lower than all other treatments (74.1 *versus* 78.9–81.4 mM/L). With gas production, the control treatment produced the most gas in bmr corn silage (283 *versus* 264–276 mL/g DM; SEM 7.2 mL/g DM) and least in corn silage (254 *versus* 279–305 mL/g DM).

4. Discussion

4.1. Effect of microbial inoculants on silage fermentation

The main goal of applying microbial inoculants is to preserve the nutritive value of the crop with minimal DM and energy losses (Muck, 1988). The lack of effect of inoculated treatments on aNDF, ADF, TN and IVTDMD compared with control was not surprising since, for example, Kung et al. (1993) reported similar effects in whole crop corn, comparing two microbial inoculant treatments with uninoculated corn. In our case, WSC were within the range in each crop required to obtain a good fermentation (Rooke and Hatfield, 2003), but natural lactic acid bacteria were at high levels (Table 1) which could have reduced inoculant effects on silage fermentation, particularly in alfalfa. The most important effects of the inoculants in our study on silage characteristics were on soluble N fractions (Table 3), rather than pH or fermentation products (Table 4). The most consistent effect was a reduced NPN concentration in LP compared to control among crops; LL was lower than control in bmr corn. The lower NPN than in the control indicates better preservation of protein in the inoculated silages. There were smaller effects of inoculants on the N fractions of alfalfa compared to bmr corn or corn, which may be related to the high levels of natural lactic acid bacteria in the alfalfa crop (Table 1).

4.2. Effect of microbial inoculants on in vitro gas production and microbial biomass yield

One of the main goals of this study was to analyze effects of silage microbial inoculants on *in vitro* ruminal fermentation of the undried silages as well as on silage fermentation. Blümmel et al. (1997a) reported that gas production was positively correlated with DM digestibility, but negatively correlated with microbial biomass yield. Based on these results, they suggested that forages that produce less gas should have better microbial biomass production. Recently, Muck et al. (2007), who conducted an *in vitro* study with alfalfa silage inoculated with one of 14 inoculants plus an uninoculated control, found that some inoculated alfalfa produced less, and some produced more, gas than did uninoculated controls, suggesting that effects of microbial silage inoculants on *in vitro* fermentation of silage are not the same among inoculants. However, they did not measure MBY.

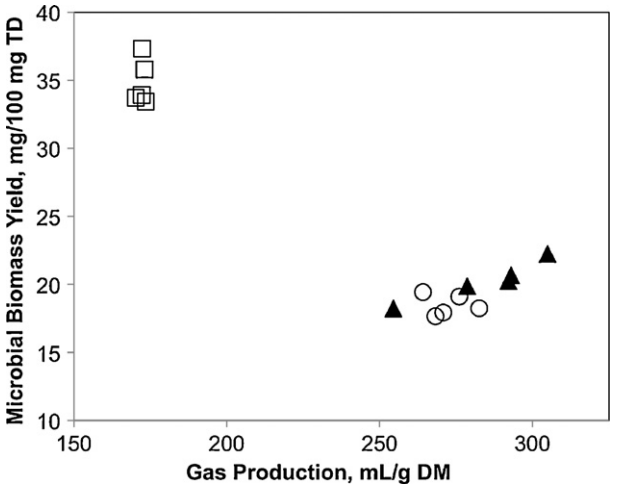

Fig. 1. *In vitro* microbial biomass yield relative to *in vitro* gas production at 48 h of incubation for alfalfa (□), bmr corn (○) and corn (▲) silages.

In our study, asymptotic gas production in inoculated alfalfa and bmr corn silages was not different from their controls (Table 5). In contrast, asymptotic gas production was higher in inoculated CS than control, the opposite of Muck et al. (2007). Among all crops at 48 h, MBY was affected by inoculant treatment with inoculants LP and LL being higher than control (Table 7), and there was no crop by inoculant interaction. Consequently, within a crop, there was no negative relationship between gas production and MBY among treatments (Fig. 1), as anticipated from Blümmel et al. (1997a). Only in comparing among the crops does one find the negative relationship between gas production and MBY, with alfalfa having the highest MBY and lowest gas production at 48 h. Thus it appears that the effect of silage inoculation on *in vitro* fermentation within a forage does not fit the pattern observed by Blümmel et al. (1997a) comparing *in vitro* fermentation among different forages.

Muck et al. (2007) reported that 65–70% of the gas produced during *in vitro* ruminal fermentation occurred in the first 9–10 h of incubation, and gas production was relatively linear, indicative of active ruminal bacterial growth. Moreover, previous results (Blümmel et al., 1997a,b) reported *in vitro* ruminal bacterial production at 24 h. In our study we estimated MBY at 9 and 48 h, and higher MBY was calculated at 9 h than at 48 h in all inoculants among crops, which is consistent with the findings of Blümmel et al. (1997b). This suggests death of some rumen bacteria by 48 h and utilization of at least a portion of those bacterial cell contents by other bacteria at 48 h. Thus 48 h is not the most appropriate time to measure MBY.

Among the three crops at 9 h, we observed that three of the four inoculant strains (*i.e.*, LP, LPe, and LL) increased *in vitro* MBY compared to control silage (*i.e.*, significant treatment effect but no crop by inoculant interaction). Effects were observed in cases where pH and silage fermentation acids were either not affected by inoculant or inoculant changes in these acids were small (*i.e.*, less than 10 g/kg DM; Table 4). In reviewing the inoculant literature, Weinberg and Muck (1996) found that there were a substantial number of cases where increases of weight gain or milk production from feeding inoculated silage occurred in the absence of effects on pH or silage fermentation products. Our results would appear to provide a hypothesis for such animal effects due to increased ruminal microbial biomass by using some silage inoculants.

In our study, the three inoculated treatments with higher *in vitro* MBY increased MBY by 8% relative to that in the control silage. If these results occurred *in vivo*, a lactating cow fed the inoculated silage would have 96 g/day more microbial CP reaching the small intestine than a cow on control silage producing 1200 g microbial CP per day. Assuming 0.67 of metabolizable protein is converted to milk protein (NRC, 2001), then the cow fed the inoculated silage would produce 64 g more milk protein or 2.1 kg milk/d assuming milk protein content is 30 g/kg milk. This analysis is predicated on milk production being limited by the supply of metabolizable protein, not net energy of lactation (NE_L). The 2.1 kg milk/d is in the upper range of the average response to inoculated silage reported by Kung and Muck (1997) in studies where the inoculated silage increased milk production, 1.36 kg milk/d, suggesting that in some cases the milk production was limited by NE_L.

While our observed increase in *in vitro* MBY is capable of explaining milk production responses to inoculated silage, the reason for the increase in MBY is uncertain. Weinberg et al. (2003, 2004) reported that lactic acid bacteria could survive in ruminal fluid, suggesting that these bacteria may directly affect ruminal fermentation in a manner that improves ruminal microbial biomass production, or perhaps these improvements in rumen microbial biomass production are mediated through inoculant effects on silage N fractions, the presence or absence of bacteriocins in silage (Gollop et al., 2005) influencing a portion of the rumen microflora, or some unknown silage characteristic or combination of characteristics. Given that total *in vitro* ruminal VFA concentration was not affected by treatment, it suggests that the increased *in vitro* MBY in the three inoculant treatments compared to those in the controls may be due to increased efficiency of silage N utilization in the inoculated treatments. Whatever the mechanism, the current study, and earlier research (Filya et al., 2007; Muck et al., 2007), suggest that animal improvement from silage inoculation may be specific to inoculant strain, and may transcend classical metrics of silage quality.

5. Conclusion

Evidence of positive animal performance effects due to the use of microbial silage inoculants has accumulated for many years, but an explanation remains unclear. We compared four inoculant treatments with uninoculated silage using whole crop alfalfa, bmr corn and corn. In these crops, the primary effects of some of the inoculant treatments were in modifying silage soluble N fractions, not altering silage fermentation acids. In addition, *in vitro* ruminal fermentation indicated that even when no differences occurred in gas production and VFA concentration between inoculated silages and control, silages from some inoculants produced higher microbial biomass yield than the control. Results suggest that some, but not all, microbial inoculants can alter ruminal fermentation and thus improve animal performance by converting more silage DM to microbial biomass. This alteration in ruminal fermentation may be linked to some combination of direct effects of specific inoculants on bacterial biomass yield in the rumen and better preservation of crop protein during silage fermentation.

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